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HORMONE PURIFICATION BY ISOELECTRIC FOCUSING IN SPACE

PREPARED FOR

NASA - GEORGE C. MARSHALL SPACE FLIGHT CENTER
CONTRACT No. NAS8-32950

BY

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JANUARY 26, 1982



(NASA-CR-161987) HORMONE PURIFICATION BY
ISOELECTRIC FOCUSING IN SPACE Progress
Report, 29 Mar. 1980 - 28 Mar. 1981 (Arizona
Univ., Tucson.) 92 p HC A05/MF A01 CSCL 22A

N82-21232

Unclas
G3/12 18166

**ENGINEERING EXPERIMENT STATION
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for the period


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Milan Bier, Principal Investigator

January 27, 1982

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REPORT ON

HORMONE PURIFICATION BY ISOELECTRIC FOCUSING IN SPACE

NASA CONTRACT NAS8-32950

A ground-based prototype of an apparatus for Recycling Isoelectric Focusing (RIEF) was designed and constructed during the first year of this Contract. The apparatus was complemented by a Multiplexed UV and pH Electronic Data Acquisition System (MEDAS). Both of these were described in details in the pertinent report to NASA and publications in the scientific literature. The present report will review the subsequent efforts under this Contract. Two main objectives were pursued:

1. The evaluation of the performance of the RIEF apparatus in ground-based operation. Special consideration has been given to the effects of gravity on its function and the determination of potential advantages deriveable from its use in a microgravity environment.
2. Development of a theoretical model of isoelectric focusing (IEF) using chemically defined buffer systems for the establishment of the pH gradients. This model was transformed to a form suitable for computer simulations and used extensively for the design of experimental buffers.

These two aspects of our work are reviewed separately in the present report. The review will be brief, as the most significant aspects of the work were published. The pertinent reprints are enclosed in the Appendix.

A. APPARATUS PERFORMANCE

I. INTRODUCTION

Electrophoresis of biological materials without the interference of gravity may become an interesting and possibly significant use of orbiting spacecraft (1). Electrophoresis is defined as the transport of electrically charged species under the influence of a direct current electrical field. Because of its singular usefulness for the analysis and separation of complex protein mixtures a number of electrophoretic techniques have been developed (2). An essential feature of these is the necessity to stabilize the liquid medium against gravity-driven convective flows. The microgravity environment of orbiting spacecraft provides an alternate means for avoidance of convection. This has been verified in pilot experiments conducted aboard the Skylab, Apollo 16, and Apollo-Soyuz space missions (3-7).

Isoelectric focusing is a particularly powerful variant of electrophoresis, in which the separation is carried out in a pH gradient. The proteins migrate, i.e., focus, to the pH region corresponding to their isoelectric point, where they become immobilized due to zero net charge. The isoelectric point of a protein is a rather characteristic parameter, and analytical IEF can resolve proteins differing by only 0.01 pH units. The pH gradient is established 'naturally', through the focusing of appropriate buffer mixtures, usually commercially available mixtures of carrier ampholytes, marketed under a variety of tradenames, such as Ampholine.

The objective of our project is the development of new technology for large scale purification of peptide hormones, proteins and other biologicals, with particular emphasis on the possible usefulness of the

microgravity environment. The basis of this technology was the development of a novel type of focusing apparatus (RIEF) utilizing a unique recycling principle. In the ground-based prototype of the apparatus (8), fluid stabilization is accomplished by a parallel array of filter elements, assuring laminarity of flow. The apparatus has been complemented by a computer controlled automated data acquisition system (MEDAS). It permits not only data acquisition in real time, but can be also used for feed-back optimization of the separation process.

Paralleling this instrument development effort, extensive computer modeling of electrophoretic processes was deemed necessary to provide a better understanding of the underlying phenomena (9, 10). This work is reviewed in a later section of this report.

The ground-based apparatus has proven to be quite useful in its own right. A multitude of samples has been processed, most of which were supplied by researchers from industry as well as this and other academic institutions, thus representing particularly difficult separations. Nevertheless, some deficiencies of the ground-based apparatus have become apparent. Briefly summarized, these are related to the use of the parallel array of filter elements for fluid stabilization, which may be avoidable in a microgravity environment. We plan to propose pilot experiments designed to clarify some critical parameters needed to permit the evaluation of the feasibility of filter-free operation. These experiments should be developed for inclusion in one of the forthcoming Shuttle flights.

II. APPARATUS

The RIEF apparatus, in its present ground-based configuration, is presented schematically in Fig. 1. The apparatus is of modular design,

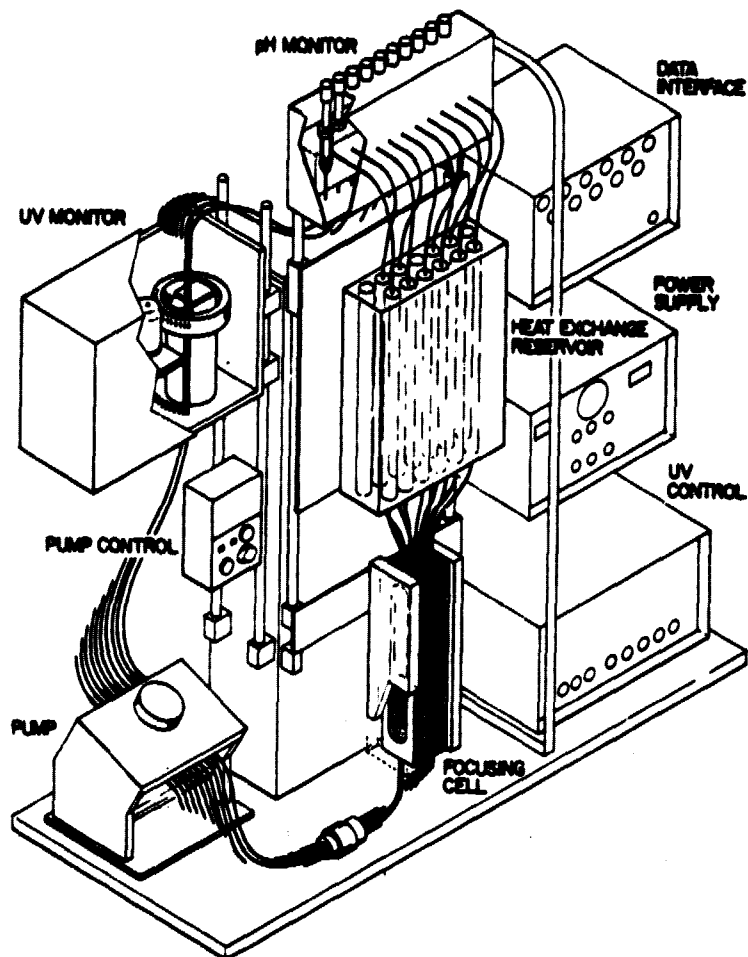


Fig. 1. Schematic presentation of the recycling isoelectric focusing apparatus (RIEF). For explanation see text.

permitting easy interchange of parts and scaling up to appropriate volumes. The protein-containing solution to be fractionated is recycled through a multicompartmented focusing cell and a multichannel heat-exchange reservoir. In each pass through the focusing cell, some migration of proteins towards their isoelectric point is obtained, thus reequilibrating the contents of the reservoirs. Finally, a steady state distribution of proteins is achieved, each protein having migrated to the reservoir closest to its isoelectric point. The Joule heat is dissipated in the heat-exchange reservoir, which also provides most of the fluid

capacity of the instrument. This method of operation permits the hypothetical scaling of the capacity of the apparatus to any desired volume, as the two critical functions of protein separation and heat dissipation have been physically separated.

The key component of the apparatus is the focusing cell. It comprises a number of parallel narrow channels, separated from each other by filter elements. These elements insure the laminar flow of the process fluid but do not impede the protein migration. The electrodes, located at the ends of the channel assembly, are separated from it by ion-exchange membranes. Recycling is accomplished by means of a multi-channel peristaltic tubing pump. The heat-exchanger comprises a series of glass reservoirs, each communicating with its corresponding channel in the focusing cell.

The apparatus is complemented by multiplexed arrays of automated pH and ultraviolet absorption sensors of our own design (MEDAS). These are under the control of a Hewlett-Packard desk top computer. The computer scales and numerically calibrates the sensors, analyzes the data statistically and can provide simple logic decisions. For calibration, two standard solutions of known pH and UV absorption are utilized. During a run, all channels of the apparatus are scanned at preset time intervals, usually 5 min., and each data point is actually an average of 25 measurements taken within a fraction of a second. While not essential for the functioning of the RIEF, these sensors provide documentation of the separation process in real time, the data being displayed graphically on a Hewlett-Packard plotter. In addition, the computer can be utilized for feed-back optimization of the separation, by regulating the withdrawal or addition of buffering fluids.

III. APPARATUS PERFORMANCE

The capacity of the apparatus is governed by the holding volume of the heat-exchanger, while the rate of separation is a function of the effective cross-sectional area of the focusing cell and the applied power. The modular design of the apparatus permits ready interchange or scaling up of either of these components. Focusing cells of cross-sectional area ranging from 1 to 100 cm² have been utilized, and further increase in area is feasible. These have fractionated up to 10 liters of protein solution in a few hours. The usual separation requires only 1-2 hrs, longer runs being necessary for larger volumes or higher resolution. While usually only ten channels are utilized, for some high resolution separations 20 channels were found to be more effective. In contrast, for the isolation of immunoglobulins from milk whey proteins, a three channel apparatus was found to be quite sufficient. Moreover, recycling is not essential, but continuous throughput could be achieved by a cascading series of several focusing cells.

The resolution achievable is mainly a function of the broadness of the pH gradient, buffer concentration, and sample composition. Usually, the pH gradient is established through the use of Ampholine or other commercial carrier ampholytes. While these are available in various pH ranges, they can readily be subfractionated in the RIEF apparatus, providing very narrow pH range buffering solutions for highest resolution. In addition, resolution can be improved by withdrawal or addition of buffering fluid during the focusing process. The computer can be instructed to analyze either the pH or the ultraviolet absorption data and regulate an auxiliary pump to withdraw, or add fluids, until the desired value of pH or absorption is reached in a given channel.

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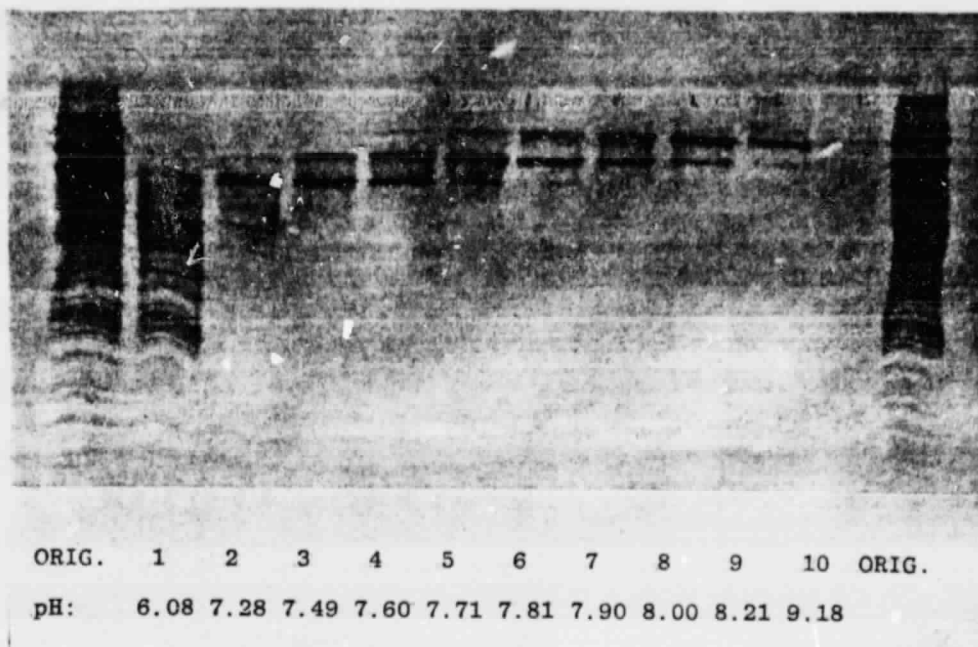


Fig. 2. Photograph of an analytical focusing gel presenting the ten RIEF fractions from a separation of clonotype rabbit antibodies to *Micrococcus lysodeikticus*. The original antibody mixture was also applied to the gel. Two major antibody components were clearly isolated.

Commercial carrier ampholytes are amphoteric polyelectrolytes produced by random polymerization of acids and polyamines. Thus, they are chemically ill-defined and their use results in contamination of the purified protein. A major effort has been made, therefore, to develop carrier buffer systems utilizing chemically well defined components, i.e., monovalent buffers, or simple ampholytes such as amino acids and dipeptides. For this purpose, it was first necessary to develop a computer model predicting the concentration distribution of these components in IEF, and describing the resulting pH and conductivity gradients. This model is fully described in a later section of this report. The predictions of the model have been verified in numerous

experiments and have been found most useful for the design of optimized buffer mixtures for specific applications. In several instances, the resolution achieved with such custom-designed buffers was superior to that obtainable with commercial carrier ampholytes.

It is not within the purview of this report to review all the experimental fractionations we have carried out. Rather, the photograph presented in Fig. 2 showing the fractionation of clonotype rabbit antibodies to *Micrococcus lysodeikticus* will suffice to demonstrate the effectiveness of the RIEF apparatus. The protein solution, contributed by Dr. L. S. Rodkey and S. Binion of Kansas State University, was fractionated in the RIEF apparatus, and the fractions obtained analyzed by conventional analytical IEF in thin layers of polyacrylamide gels. It is evident that a very narrow cut of antibodies has been isolated. Similar separations have been carried out on various enzymes, peptide hormones, interferon, hemoglobins, blood and milk proteins, etc. As previously stated, most of these samples were furnished by outside collaborators and represented rather difficult separations.

IV. CONCLUSIONS

The importance of protein purification technology has acquired a new dimension as a result of the recent achievements in the field of genetic engineering. Recombinant DNA techniques have resulted in the production, through bacterial fermentation, of some biologicals of major pharmaceutical importance, such as insulin and interferon. Hybridoma techniques have provided another series of important biologicals, the monoclonal antibodies. In both techniques, however, the desired product is contaminated by proteins derived from the host organism, the bacterial

cell or the mouse myeloma cell, respectively. The purification of the desired protein is essential if it is to be used as a pharmaceutical, and this is often a most laborious task. A similar situation prevails in the production of peptide hormones through solid phase synthesis. Incomplete reaction at various steps in the synthetic process results in the production of analogues which may have antagonistic properties and which must be eliminated.

In general, purification is accomplished by the artful sequencing of various fractional precipitation processes, chromatographic techniques and affinity methods. For quality control in purification, however, only electrophoretic techniques can provide objective evidence of purity, due to their superior resolution. Thus, there have been numerous efforts to develop electrophoretic instruments suitable for large scale preparative purposes. Two main obstacles to the scaling up of these instruments have been encountered: the need to dissipate the Joule heat generated by the electrical current, and the apparent need to stabilize the fluid column against convective disturbances. As a result, electrophoresis is still limited to analytical or micropreparative applications.

The previous limitations to the scaling up of electrophoretic processes have been overcome in the KIEF apparatus. It represents the first preparative apparatus having both high resolution and high volume throughput. Thus, we feel that it fulfills an important requirement of modern protein purification technology. In fact, the KIEF apparatus has been incorporated in a pilot plant for the purification of a human interferon produced using recombinant DNA techniques by the Schering Corp. of Bloomfield, N.J.

Even though effective, the ground-based prototype has some limitations,

mainly due to the necessity of utilizing the filter elements for the stabilization of liquid flow through the apparatus. Briefly summarized, these are:

1. The filter elements generate an electroosmotic pressure gradient between successive RIEF compartments. This gradient causes back-flow of fluid, thus causing loss of resolution.

2. This filter-caused electroosmosis is highly unpredictable as it largely depends on the type of protein present in the system. This is due to adsorption of proteins on the surface of the filter material. In certain cases (Armour ACTH and bacteriophages), this adsorption was sufficient to cause serious loss of solute being fractionated. A number of different filter elements has been investigated to find the least adsorptive ones, and at present we usually use monofilament screens of nylon, with a nominal porosity of 6 microns. Overall, these have given us the least electroosmosis, but are still far from being perfectly satisfactory in all systems.

3. Many proteins tend to precipitate at their isoelectric point. Precipitation can be avoided by the addition of high concentrations of urea, but this may cause loss of biological activity of some proteins due to denaturation. In such a case, RIEF fractionation is not possible. Far more often, however, there is only a slight precipitation, which would not affect the fractionation, except that it may cause fouling of the filter elements. This accentuates electroosmosis and causes frequent abortion of experiments.

Microgravity may provide an alternative approach to RIEF fractionation by suspending the need for the filter elements. Elimination of these elements

in the presence of terrestrial gravity inevitably results in drastic convection and rapid mixing of the sample.

Saville (11) has developed a computer model analyzing fluid stability in continuous flow electrophoresis instruments. This model predicts the observed fluid instability in instruments of similar design as the RIEF. It also predicts that these instabilities may be eliminated in a micro-gravity environment.

Unfortunately, the operation of any electrophoretic apparatus is influenced not only by gravity-caused convection, but also by gravity-independent electroosmosis. Electroosmosis is caused by the electrical charge of the medium within which electrophoresis is carried out, this charge causing a flow of fluid usually in a countercurrent direction to the sample migration. One needs to differentiate between two types of electroosmosis: 'wall' electroosmosis is caused by the charge of the walls of the vessel, while 'porous plug' electroosmosis is caused by the presence of any porous obstruction in the migrating pathway. The filter elements in the RIEF cause the porous plug type of disturbance, but seem to abolish the wall effects. By eliminating the filters, the wall effects may become dominant.

An adaptation of the Saville model to continuous flow IEF has shown the particular vulnerability of IEF to electroosmosis. During focusing, the mobility of proteins asymptotically approaches zero, while electroosmosis does not. Thus, a disruption of the focusing process may result.

Electroosmosis is readily measurable in systems with uniform buffer composition. This is not the case in IEF, which is characterized by a continuous pH gradient. All ground-based methods for IEF require fluid stabilization against convection, which simultaneously suppresses wall

electroosmosis as well. Thus, no experimental data are available on the extent of electroosmosis in IEF. The microgravity environment provides the unique possibility of eliminating gravity-driven convection without affecting electroosmosis.

In the coming program year we plan to develop a rather simple experimental apparatus to separate the effects of gravity and electroosmosis aboard a Shuttle flight. A series of focusing columns (0.25" x 2") will be loaded with two colored proteins, hemoglobin and blue-stained albumin, and photographed at predetermined time intervals after application of a d.c. field. The columns will differ in the method used to suppress electroosmosis. It is hoped that this apparatus will be included in a forthcoming Shuttle flight and will provide the crucial information as to the feasibility of continuous flow focusing in space.

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B. COMPUTER SIMULATION STUDIES

I. INTRODUCTION

Isoelectric focusing (IEF) is widely used for the analysis of proteins because of its exquisite resolution. IEF is based on the electrophoretic transport of amphoteric sample components to their respective isoelectric points (pI) in a stable pH gradient. Svensson (1, 2) was the first to show the usefulness of IEF, by generating stable pH gradients through the focusing of a complex buffering mixture of polyamino-polycarboxylic acids. This buffer was synthesized for this specific purpose by random polymerization (3) and introduced commercially by LKB Produkter of Bromma, Sweden, under the trade name Ampholine.

Svensson (1) also developed the theory of IEF of a protein sample assuming the pre-existence of a stable linear pH profile and uniform conductivity. Ampholine-generated pH profiles approximate these assumptions fairly well. However, the effectiveness of Ampholine has, in some sense, stymied the development of theories dealing with the establishment of the pH gradient, since the composition of Ampholine is unknown.

Ampholine and other similar commercial buffers are excellent for analytical purposes, but are unacceptable for many preparative tasks as they result in contamination of the purified protein by a chemically and biologically ill-defined mixture. Thus, there have been numerous empirical attempts to develop stable pH gradients using mixtures of chemically well-defined amphoteric or non-amphoteric buffers (4-9). These efforts were largely unsuccessful, due in part to the absence of a readily applicable theory dealing with the establishment of the pH

profile. The only theoretical treatment of the problem is that of Almgren (10), but some of his simplifying assumptions prevent the use of his model in practical situations.

Our interest in a more adequate theory of steady state IEF was stimulated by the development of the RIEF apparatus, described in the first section of this report. The apparatus has overcome previous limitations on the quantity of material that can be processed by IEF, thus conveying a new degree of urgency to the development of chemically well-defined buffers. It was felt that an empirical approach would be no more successful than those of prior researchers and that a better understanding of the underlying phenomena was essential.

A major effort was undertaken to develop and apply a theory defining the steady state characteristics of pH gradients generated by biprotic ampholytes or monovalent buffers (11, 12). The assumptions mentioned were eliminated in our model based upon relationships describing the electric field, the diffusional current, the components' chemical equilibria, and the mass transport resulting from diffusion and electromigration. This model is capable of accepting the characteristic electrochemical properties of constituent ions, including differences in mobilities of the positively and negatively charged species of each ampholyte. The theory has permitted us to carry out extensive computer simulations of IEF.

II. BASIC CONCEPTS

The model for the steady state in an IEF system with L biprotic ampholytes (A_j) has been described in detail (11, 12). At present, we only wish to show the underlying basic concepts:

Dissociation Reactions

The usual formulation is employed. The constant K_w characterizes the dissociation of water and can be represented by the relation

$$K_w = [H^+] [OH^-] \quad (1)$$

while the dissociation of biprotic ampholytes is given by the equilibria

$$K_{j1} = \frac{[A_j^0] [H^+]}{[A_j^+]} \quad (2)$$

$$K_{j2} = \frac{[A_j^-] [H^+]}{[A_j^0]}$$

where K_{j1} and K_{j2} denote dissociation constants.

Diffusion and Electromigration

The flux F (mole/m²s) of the i -th species caused by electromigration and diffusion is given by

$$-F_i = \Omega_i z_i M_i \frac{\partial \phi}{\partial X} + \frac{RT}{e} \Omega_i \frac{\partial M_i}{\partial X} \quad (3)$$

where e is the molar charge or Faraday constant (96500 Coulombs/mole), ϕ - potential (V), R - universal gas constant (8.314 kg.m²/s².°K.mole), T - absolute temperature (°K), X - distance from one end of the column (m), and Ω_i - mobility coefficient (m²/V.s), z_i - valence, M_i - concentration (mole/m³), of i -th species. The Einstein relation between mobility coefficient (Ω_i) and diffusion constant (D_i):

$$D_i = \frac{RT}{e} \Omega_i$$

shows how equation (3) is derived.

Conservation of Mass

The law of mass conservation of i-th species in the absence of bulk flow is:

$$\frac{\partial M_i}{\partial t} = - \frac{\partial F_i}{\partial x} + R_i \quad (4)$$

where R_i denotes the rate of production of the i-th species (mole/m³.s), and t is time (s). The net production of i-th ampholyte is zero, i.e., the sum of R_i for the three possible species of each ampholyte, defined in equation 2, is equal to zero.

Conservation of Charge

There is no net production of charge at any point of the column, except at the electrodes, and this can be written as

$$e \sum_{i=1}^{3L+2} z_i R_i = 0 \quad (5)$$

Electroneutrality

Electroneutrality can be assumed on the relevant physical scale and is given by the relation

$$\sum_{i=1}^{3L+2} z_i M_i = 0 \quad (6)$$

III. COMPUTER SIMULATION STUDIES

Computer simulation of IEF requires the input of the physical dimensions of the column, the applied current and the mobility coefficients and dissociation constants of the components. The computer output includes the concentration profiles along the column axis of all species of all components, including hydrogen and hydroxyl ions, the pH profile, as well as the profiles of conductivity and potential. Rather

than describing in detail all the simulations, some of which were published (11, 12), we wish to present only the general approach utilized.

Simulation studies with idealized ampholytes

Idealized ampholytes are hypothetical compounds which have been assigned arbitrary mobility coefficients and dissociation constants. Our model has permitted the study of the effects of a systematic variation of each parameter, taken one at a time or in concert. The parameters which have been investigated comprised all of the input variables listed above. In addition, two studies have been carried out which are of particular interest to the experimentalist:

i. The effects of varying the spread between the isoelectric points (ΔpI) of ampholytes at constant ΔpK . Three binary mixtures were compared, with ΔpI of 0.5, 1 and 2 pH units, symmetrically distributed around pH 6.5. The ampholytes were assigned a $\Delta pK = 2$, and mobilities of $3.0 \times 10^{-4} \text{ cm}^2/\text{V.s.}$

ii. The effects of varying the spread between the dissociation constants of the ampholytes (ΔpK) at constant ΔpI . Again, three binary mixtures were compared, with ΔpK of 1, 2 and 4, at ΔpI of 1, the other electrophoretic parameters remaining unchanged.

Simulation studies with real ampholytes

Simulations with idealized compounds have shown that comparable pH and total ampholyte concentration gradients are obtainable from a hypothetically infinite number of ampholyte pairs, by varying the ΔpK . Significant differences were seen, however, in concentration profiles of charged species, resulting in variations of conductivity and potential profiles. Certain rules for matching components in binary mixtures were formulated. Unfortunately, in practice only a very limited number of

ampholytes are available, mainly amino acids and di- or tri-peptides. Even fewer are the number of ampholytes for which reliable data on dissociation constants and mobility coefficients are reported in the literature.

A large number of simulations has been carried out with two and three component mixtures, using the available literature data. Simulation revealed some interesting and intuitively unpredictable facts. For example, simulated focusing of a mixture of aspartic acid ($pK_1 = 1.88$, $pK_2 = 3.65$, $pI = 2.76$, mobility = $3.23 \times 10^{-4} \text{ cm}^2/\text{V.s}$) and m-aminobenzoic acid ($pK_1 = 3.12$, $pK_2 = 4.74$, $pI = 3.93$, mobility = $3.01 \times 10^{-4} \text{ cm}^2/\text{V.s}$) gave nearly linear pH gradients and the concentration profiles were quite symmetrical. Thus, they seemed to meet the requirements for a useful ampholyte pair for experimental focusing. Quite different results were obtained, however, if m-aminobenzoic acid was replaced by glutamic acid ($pK_1 = 2.19$, $pK_2 = 4.25$, $pI = 3.22$, mobility = $2.97 \times 10^{-4} \text{ cm}^2/\text{V.s}$), even though their electrochemical parameters appeared rather comparable. With this pair, simulation predicted that the concentration of glutamic acid goes through a distinct maximum, falling off towards the anode.

Such studies have proven the usefulness of our model for the selection of ampholytes for the establishment of useful pH gradients. The following general rules can be formulated:

1. To obtain near linear pH profiles, components should be chosen having a ΔpK in the range of 2 to 4 and a ΔpI of about 1 pH unit.
2. Obviously, any dissociable group will buffer only within approximately one pH unit on either side of its dissociation constant. In the focusing of a pair of ampholytes, only the basic group of the more

acidic ampholyte and the acidic group of the more basic ampholyte will exert a buffering effect within the pH range defined by their ΔpI . Thus, it is important that the difference between the pK_2 of the acidic component and the pK_1 of the more basic component be less than 2 pH units. The importance of this factor, to which we refer as the cross- ΔpK , was not previously realized, though it can be easily understood, once revealed by simulation.

IV. DISCUSSION

The development of the mathematical model of IEF, briefly presented in this report, is of more than academic interest for our project. In fact, we consider it essential for the development of the technology of peptide and protein purification by IEF. In analytical IEF, the pH gradients are generated by chemically ill-defined buffer mixtures, available in commerce under a variety of tradenames, such as Ampholine. These are unacceptable in preparative work, as they result in product contamination.

The prediction of our simulations were verified in numerous experiments on focusing of albumin-hemoglobin mixtures, fractionation of snake venoms, human hemoglobins, milk whey proteins, synthetic ACTH hormones and others. In many cases, the resolution achieved with these computer-selected buffer components was actually better than that obtainable in Ampholine. Nevertheless, for general analytical purposes, Ampholine-like buffers have the advantage of linearly covering broad pH ranges. At the present time computer-derived buffer systems have to be custom-optimized for each protein to be separated. Eventually, as more experience is gained, this limitation may be overcome.

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LARGE-SCALE RECYCLING ELECTROFOCUSING

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INTRODUCTION

Electrofocusing is generally recognized as a powerful method for the analysis and micropreparative separation and purification of various biological materials, including proteins, peptides, nucleic acids, viruses, and even some living cells. Although it is a relatively new variant among the electrophoretic techniques, it has gained widespread acceptance as a result of the commercial introduction of Ampholine, a buffer system that allows easy establishment of stable pH gradients.

Numerous efforts have been directed toward augmenting the methodology of electrofocusing, and this literature has been summarized in several recent symposia¹⁻³. In batch instruments, gradients are usually stabilized by sucrose density gradients or polyacrylamide gels (PAGs). The capacity of such instruments is limited to about 1-10 mg/cm² apparatus cross-area for each protein fraction. Apparatus cross-section cannot be enlarged at will because of the need to dissipate the Joule heat generated by the electric field.

Thus, for large-scale preparative work, continuous-flow instruments seem to be essential. Unfortunately, continuous-flow electrofocusing in free solutions is plagued by severe distortions of boundaries of separating materials caused by: 1) the parabolic nature of liquid flow through confined channels due to viscous drag (flow is fastest through the center of the channel and decays parabolically toward the walls); 2) electroosmotic flow at the walls superimposes a second type of parabolic flow on the liquid, in a direction normal to that of the parabolic distortion caused by viscosity; 3) density gradients that arise from the temperature and sample concentration can cause convective flow of fluid, completely disrupting the separating boundaries; and 4) temperature gradient from center to the cooled walls of the apparatus cause differential migration rates. These disruptive factors in continuous-flow instruments have been discussed extensively.^{4, 5}

To overcome these difficulties in continuous-flow electrofocusing, Fawcett⁶ stabilized the fluid flow by porous support or by sucrose density gradients. Both approaches gave comparable results, but the throughput

obtained with either method was limited to about 300-500 mg of protein sample per day and was therefore comparable with that obtainable in static systems.

In this chapter we describe a new approach to fluid stabilization and increased throughput. Stabilization is achieved by allowing the process fluid to flow uniformly through an array of closely spaced filter elements that are oriented parallel to the electrodes and parallel to the direction of flow. This technique seems to overcome the major difficulties of parabolic flow and electroosmosis at the walls, while it limits the convection to the chamber subcompartments that are defined by adjacent spacers. Increased throughput is achieved by recirculating the process fluid through external heat-exchange reservoirs, where the Joule heat is dissipated. In most other methods, it is the requirement for heat dissipation that limits the size and capacity of the instruments. In each pass through the focusing apparatus, only a partial shift toward eventual equilibrium focusing is achieved, but through repeated recycling a final steady state is obtained. Thus, this approach to large-scale separation is possible only with a focusing technique, and not, for instance, with zone electrophoresis, where no stable end point is reached.

Many types of membrane- or filter-subdivided electrophoresis instruments have been described. Early versions have been proposed by Theorell,⁷ Tiselius,⁸ and others, and this literature has been reviewed in details.⁹ A more recent apparatus, specifically designed for electrofocusing,¹⁰ contained ways to cool each subcompartment internally. All of these instruments were designed essentially for batch-type operation--i.e., all fluid to be separated is contained within the apparatus. Thus, relatively large internal volumes were required. In the present apparatus, it is advantageous to minimize the internal volume of the apparatus by reducing the space between adjacent filters as much as possible.

The present apparatus bears closest resemblance to the apparatus used for forced-flow electrophoresis,^{11, 12} where the same principle of external cooling is used. In forced-flow electrophoresis, a repeating array of membranes and filters is used, and only a single isoelectric fraction is obtained by cross-flow electrophoresis rather than equilibration.

APPARATUS AND METHODS

The essential features of the focusing apparatus are presented schematically in Figure 1, (cross-sectional view). The apparatus has 10 input and

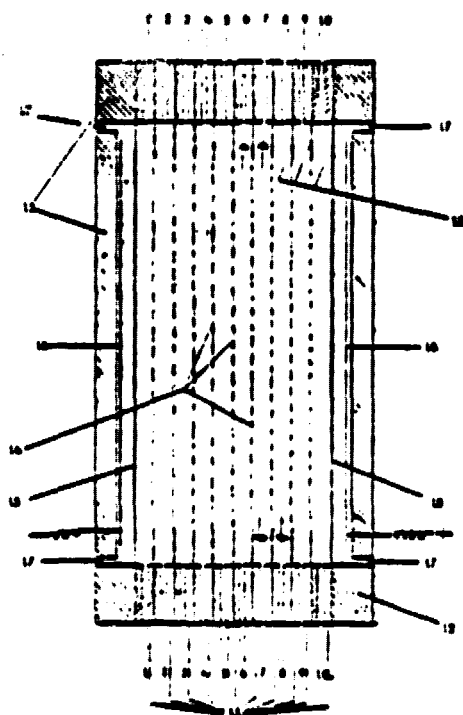


Fig. 1. Schematic cross-sectional view of the focusing apparatus. Ten Plexiglas spacers with inlet and outlet ports are separated by filter elements, and sandwiched between end plates that contain recessed electrodes. Membranes isolate the electrode compartments from the spacers.

output ports that are located at the opposite ends of Plexiglas spacers, separated from each other by filter elements. These filters have to allow free flow of water and transport of solutes. At present we are using poly(vinyl chloride) filters with a nominal porosity of 5 μ , but other comparable microporous filters could be equally well used. The filter-spacer assembly is tightened between two Plexiglas end plates, housing recessed platinum electrodes. The electrode compartments also have ports for recirculating electrolytes and are separated from the spacer by membranes. These membranes should be different from the filters because they should inhibit free flow of liquid or transport of solutes, other than small ions. Two types of membranes were used: dialyzing regenerated cellulose membranes and ion exchange membranes. The internal dimensions of the spacer and electrode cavities are 2 x 20 cm, and each spacer is 0.25 cm thick. Although we are using only 10 spacers now, it is obvious that any number could be used, depending of the number of fractions desired. There is also nothing critical about the dimensions of the spacers, except that their area should be kept rather large and their thickness as small as possible.

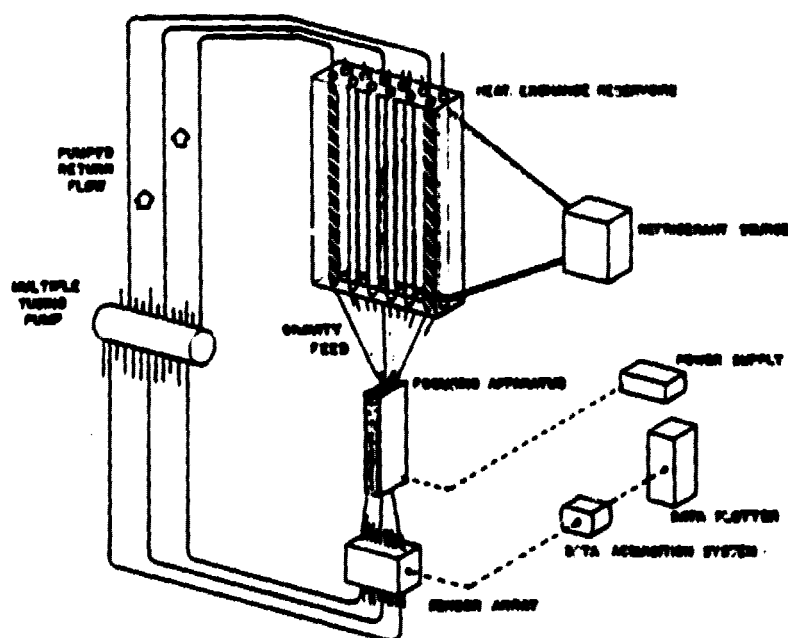


Fig. 2. Schematic presentation of the recycling apparatus. Heat-exchange reservoirs are cooled by brine circulation, and their contents are fed by gravity to the focusing apparatus described in Figure 1. A multichannel peristaltic pump returns all the fluids to the reservoirs. Presently under development is the sensor array for automated pH and ultraviolet absorption measurements. This array will be interfaced with a Hewlett-Packard desk-top calculator and data plotter.

Operation of the system is best explained by Figure 2. The fluid to be focused is subdivided into the individual glass reservoirs contained within the plastic enclosure, through which a 4°C cooling brine is recirculated. Twelve glass reservoirs are presently used, with a capacity of 100 ml each; 10 of them communicate with the corresponding filter-defined subcompartments in the focusing apparatus, and two communicate with the electrode compartments. The fluid flows by gravity from the reservoirs into the focusing apparatus and is returned to the reservoirs by a multichannel peristaltic pump. Presently under construction is an array of pH and ultraviolet absorption sensors, which we plan to interface with a desk-top calculator for automatic data collection and possible feedback control. Figure 3 is a photograph of the apparatus.

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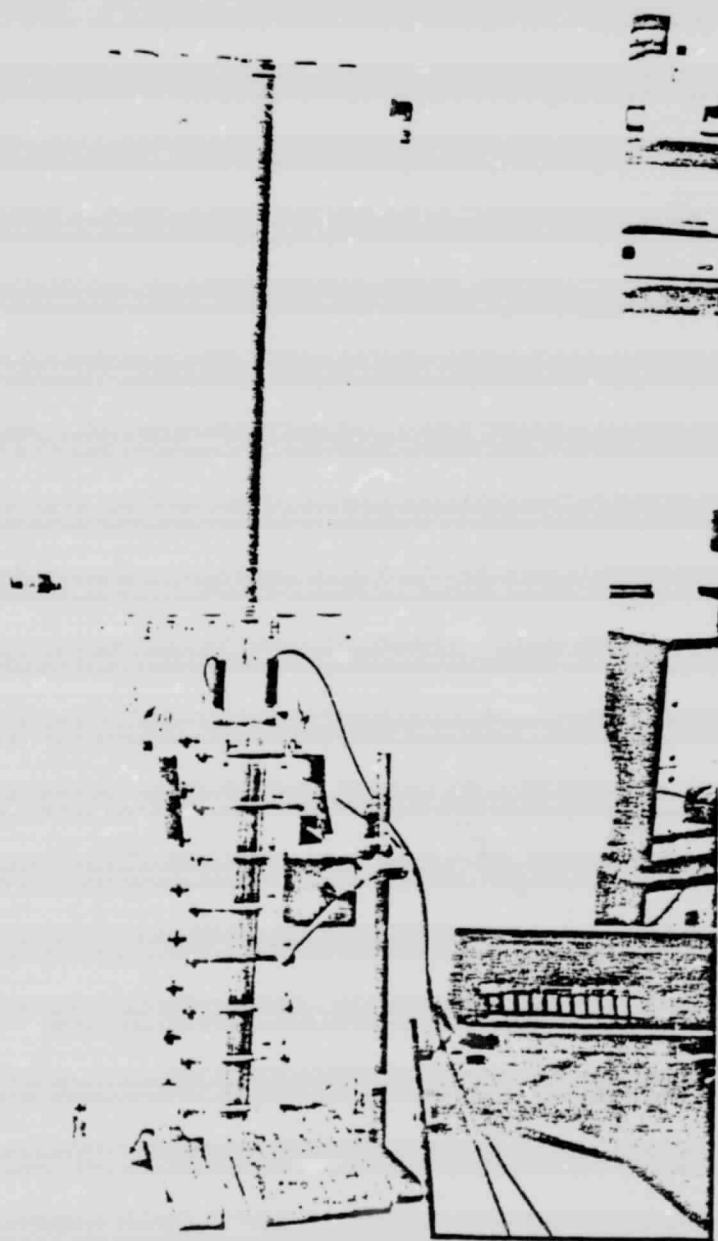


Fig. 1. The heat-exchange reservoirs and focusing apparatus, with some peripheral equipment.

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The performance of this system depends, of course, on the performance of the focusing apparatus. The usual disturbances of boundary profiles in free solution continuous-flow electrophoresis have been enumerated. In the present apparatus, the array of filters eliminates the problem of the parabolic flow profile due to viscous drag, at least in the direction of the electric field. In fact, the filter array imposes a semblance of plug flow to the moving liquid, plug flow being characterized by equal flow velocity across the entire cross-section of the apparatus. Better plug flow is theoretically obtained by porous support media, but their packing is rarely uniform enough to allow rapid uniform flows. Experimentally, we have also not been able to see any electroosmotic flow at the walls, by using visibly colored solutes, such as hemoglobin. The filters themselves do cause some electroosmosis,¹² but this is uniform across the whole filter area and does not seem to be troublesome in the present mode of operation. Because the cell is not cooled, there are no lateral temperature gradients within it, and the filters seem to confine any density-caused convective flows to each subcompartment. Thus, the mode of operation of the apparatus does not seem to be affected by the insertion of turbulence-causing plastic grids into each subcompartment. Obviously, however, no continuous pH gradient can be established, only a step gradient across each filter. The magnitude of each step will depend on the number of spacers used and the pH range of Ampholine used to make up the buffer. The performance of the apparatus is also relatively independent of the flow-through velocity, which is maintained rather high (about 10 ml/min/channel) to minimize heating of the process fluid in each pass.

Two modes of operation are possible: either the sample is added with the desired Ampholine immediately at the start of the focusing experiment, or the Ampholine is prefocused, and the resulting pH gradient is determined. The sample can then be added to the reservoir of the desired pH value, thus avoiding exposure of the sample to extreme pH values. This second mode of operation also decreases the time of sample processing because the buffer has been prefocused. During the run, aliquot samples can be withdrawn and the pH and concentration profiles can be determined. When stabilization is reached, the fractions can be collected by disconnecting the return lines from the heat-exchange reservoirs, and redirecting the flow into appropriate reservoirs, without interruption of the electric current. Monitoring of the pH and concentration changes will be greatly facilitated once the automated sensor array is fully developed.

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RESULTS

The performance of the apparatus can best be documented using colored proteins, such as hemoglobin and albumin stained with bromphenol blue. The sequence of events is shown in Figure 4. The Ampholine solution (2 ml

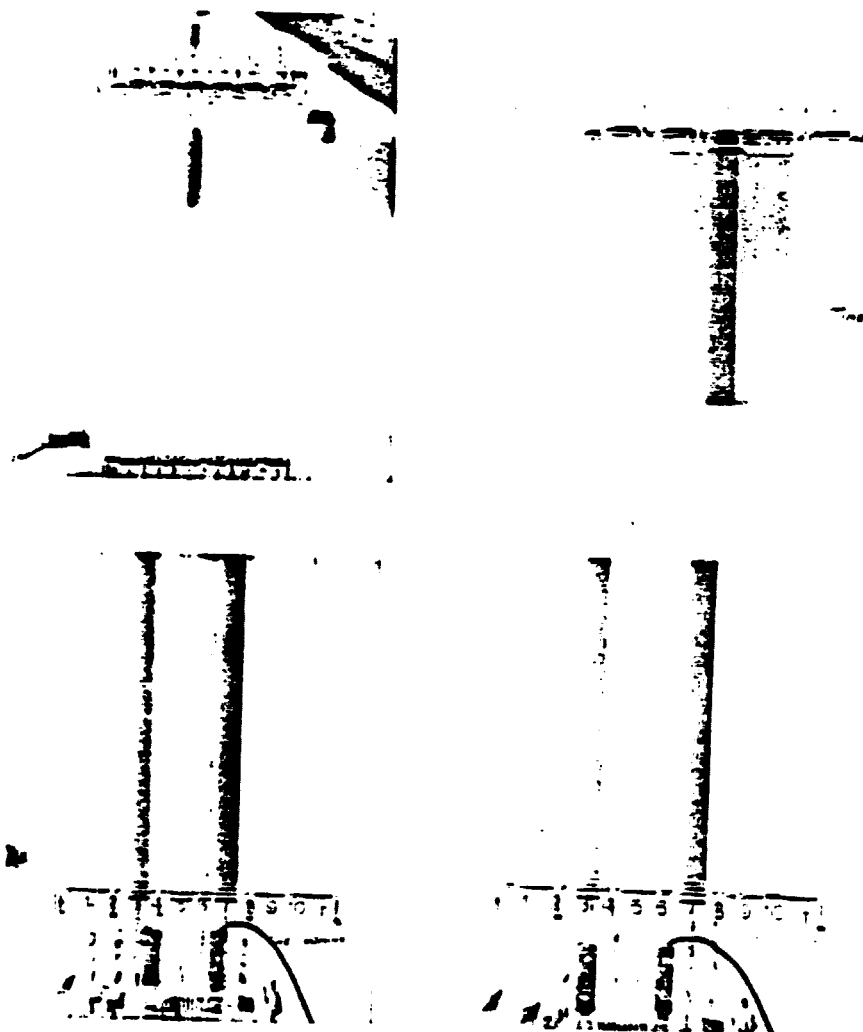


Fig. 4. Sequence of photographs showing the introduction of a mixture of blue stained albumin and hemoglobin into a central reservoir, and its subsequent stages of separation at 15-, 60-, and 120-min recycling times.

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Ampholine, pH 3.5-10) was prefocused for 2 hr in the apparatus, and the mixed hemoglobin-albumin solution was introduced into a central reservoir. The sequence of photographs shows the progressive separation of hemoglobin and stained albumin as recycling is continued. Visibly stable conditions have been achieved within 120 min, at a constant 200 V. The starting current is about 100 mA, gradually decreasing to 19 mA at the end of the run. Thus, with a focusing time of 4 hr and a sample focusing time of 2 hr, a total of 1 liter of fluid containing 0.4 g each of albumin and hemoglobin has been separated.

This separation has been repeated by avoiding prefocusing of Ampholine, and the apparatus was initially loaded with both Ampholine and the two proteins. Essentially identical protein distribution was obtained within 2 hr of focusing. Figure 5 shows the pH profiles obtained with the prefocused Ampholine alone and in the above two runs. The data show the good repro-

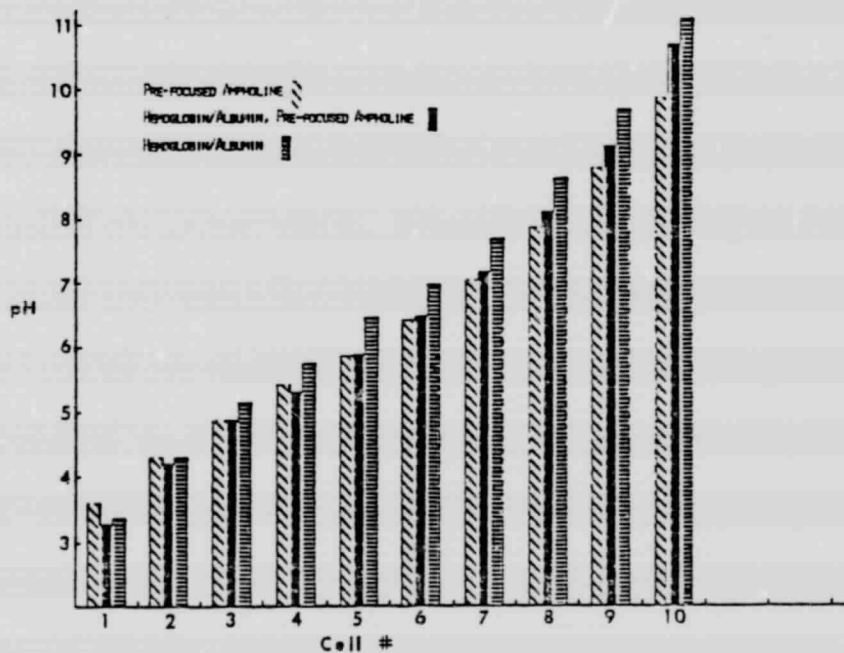


Fig. 5. Profiles of pH values in the 10 reservoirs obtained by: 1) pre-focusing the 3.5-10 pH range Ampholine, 2) refocusing the mixture of albumin-hemoglobin, and 3) focusing the same mixture of Ampholine and proteins without Ampholine prefocusing.

ducibility of the results, and we do not know if the small differences are caused by artifacts or by the method of operation.

The above separation is rather trivial because the isoelectric points of hemoglobin (ca. pH 7.4) and albumin (ca. pH 4.8) are so disparate. For more critical separations, we adopted a solution of dialyzed egg whites, from which all proteins that precipitate in distilled water were eliminated by centrifugation. On analytical gel electrophoresis, this protein mixture shows a main band at about pH 4.8, corresponding to ovalbumin, and a series of other minor bands extending to pH 7.0. A sharp lysozyme band is also seen at pH 10. This protein has been submitted to focusing, at a concentration of 2g/liter, in Ampholine ampholytes of various pH ranges. The results are shown in Figures 6-9. The top part of each diagram reproduces the patterns obtained on analytical focusing of each fraction in PAGplates using the broad-range Ampholine (pH 3.5-10) as well as the patterns obtained with the original protein mixture. The bottom diagram shows the pH profile and the protein-

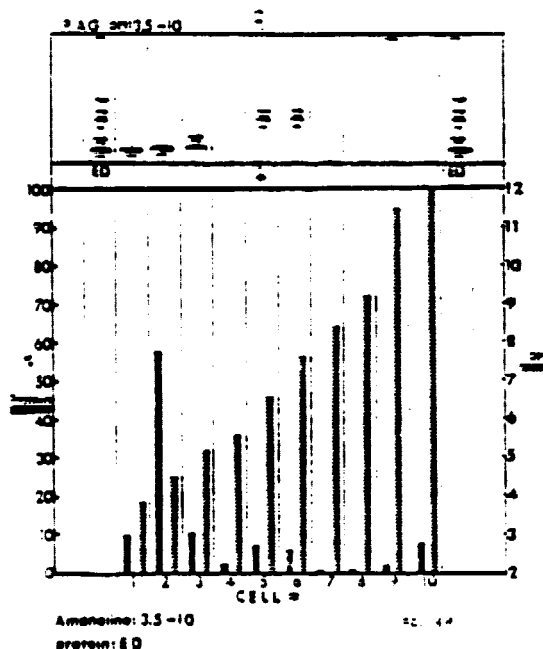


Fig. 6. Focusing of dialyzed egg-white proteins in Ampholine, pH 3.5-10. Top: analysis of the fractions in polyacrylamide gel plates, the two extremes showing the original protein sample. Bottom: pH profile and protein distribution in the 10 reservoirs.

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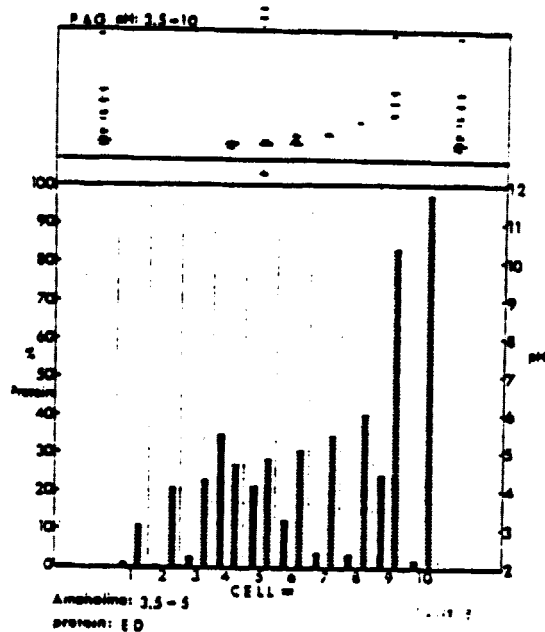


Fig. 7. Focusing of dialyzed egg-white proteins in Ampholine, pH 3.5-5. Data are presented as in Figure 6.

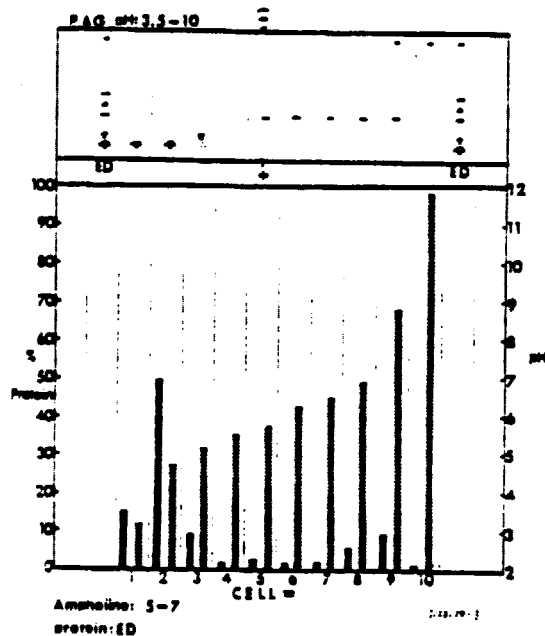


Fig. 8. Focusing of dialyzed egg-white proteins in Ampholine, pH 5-7. Data are presented as in Figure 6.

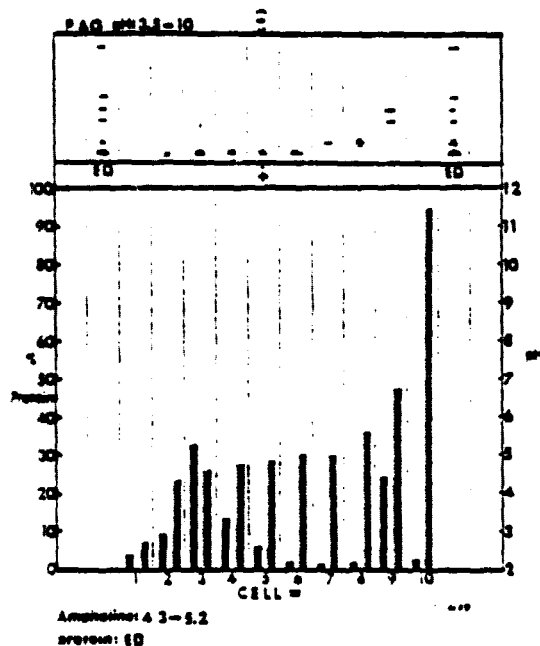


Fig 9. Focusing of dialyzed egg-white proteins in Ampholine, pH 4.3-5.2. The Ampholine with this pH range was prepared from a broad-range Ampholine in the focusing apparatus. Data are presented as in Figure 6.

distribution profile in the 10 fractions. The protein-distribution profile has been determined spectrophotometrically at 280 nm.

The data clearly show that excellent separation of some minor focusing bands has been obtained by recycling focusing, using the same procedures as described above for albumin-hemoglobin. No assignment of bands to specific known egg-white proteins was attempted. As expected, the shallower the pH profile, i.e., the narrower the pH range of Ampholine chosen, the better the separation. The data also show that with narrow-band Ampholine, the extreme reservoirs, i.e., those closest to the two electrodes, escape proper pH control and are more acidic and basic than covered by the Ampholine range.

Of particular importance is the experiment reproduced in Figure 10. The focusing was attempted in the complete absence of any Ampholine or other pH-stabilizing buffer system, other than the protein itself. Ampholine ampholytes are very efficient for stabilizing the pH gradient but have some undesirable properties, principally as they result in the contamination of the final product with this product of uncertain composition. Their cost is also

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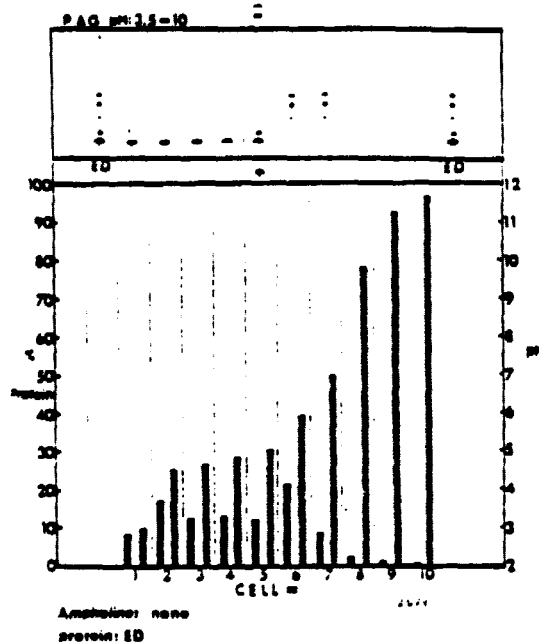


Fig. 10. Focusing of dialyzed egg-white proteins in the absence of ampholine or any other buffer, except the protein itself. Data are presented as in Figure 6.

prohibitive for truly large-scale industrial applications. Thus, it is significant to have at least some degree of separation in the complete absence of any buffer system.

DISCUSSION

A new method for large-scale electrofocusing has been described, based on recycling continuous flow, with stabilization of the fluid flow by filter elements. The apparatus is capable of large-scale application because the throughput is independent of the capacity of the focusing apparatus and depends only on the size of the heat-exchange reservoirs and the time given to recycling. Obviously, if one wishes to minimize this time and to increase the throughput beyond that described here, larger-filter cross-sections should be used. With the present cross-section of 20 cm^2 , rapid focusing of 1-liter volumes has been demonstrated. In a preliminary experiment using membrane areas of 100 cm^2 , 8 liters of hemoglobin and albumin mixtures were focused in an overnight run. The present apparatus gives only 10 fractions,

but this number can be increased easily by increasing the number of spacer-filter combinations, defining each subcompartment, and correspondingly increasing the number of reservoirs. We are presently in the process of assembling a 48-compartment apparatus for more critical separations.

Obviously, here we have not fully documented the usefulness of this apparatus. We deliberately used only proteins that do not precipitate at their isoelectric point. Such precipitation may prove to be disastrous in terms of fouling up the filters, clogging entry and exit ports, and so forth. We also do not know what effect the electroosmotic flow caused by the filter elements will have on the resolution. There is also a significant density gradient across each filter element if large differences in protein concentration are obtained in adjacent compartments, as shown in the hemoglobin-albumin separations. We do not know the maximum concentration differentials that can be tolerated, and in some egg-white experiments the concentration in individual compartments exceeded 1%. It is expected that this would cause some gravitational instability and convective flow across the filters, as obtained in electrodecantation and forced-flow electrophoresis. Both of these problems, i.e., precipitation of isoelectrically insoluble proteins and gravitational flow across the membranes, may be alleviated in a gravity-free environment, such as in orbiting spacecraft.¹³

Large-scale preparative use of electrofocusing is also complicated by the present necessity of stabilizing pH gradients by Ampholine ampholytes. This step requires subsequent separation of Ampholine from the purified product. With proteins, the separation can be done by chromatography on Sephadex, but with smaller peptides, such separation may not be possible. The separation done in the absence of Ampholine or any other buffering agent except the protein itself, may represent one possible approach. We are now studying other approaches, based on establishment of pH gradients by amino acids and peptides, somewhat along the lines previously used in isotachopheresis.^{14, 15} Preliminary data on the theoretical distribution of these compounds in an electric field are the subject of a separate paper by Allgier et al.¹⁶

It is evident that replacement of Ampholine with other buffering agents will require extensive experimental work to establish pH profiles and their stability. Thus, we are developing an array of pH and UV absorption sensors. These will be used with a Hewlett-Packard desk-top calculator and X-Y plotter for automatic rapid data collection and possible feedback control. We feel confident that the development of this complex system will permit optimization of the process in an efficient manner.

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Although the possibility of eliminating Ampholine ampholytes still remains to be proved, the method has the distinct advantage of at least avoiding possible trace contaminants arising from the use of density gradient-forming additives, porous support media, or polyacrylamide gels, none of which are needed in the present system. The throughput of the apparatus can probably be increased to commercially meaningful quantities.

ACKNOWLEDGMENT

This work has been partially supported by NASA grant NSG-7333.

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Reprinted from **PEPTIDES: Structure and Biological Function**
Proceedings of the Sixth American Peptide Symposium
Edited by E. Gross and J. Meienhofer
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NEW DEVELOPMENTS IN ISOELECTRIC FOCUSING

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Introduction

Isoelectric focusing (IEF) is generally recognized as a powerful method for the analysis and micropreparative separation and purification of proteins and larger peptides. In this paper we wish to report on our multidisciplinary effort to develop a new system for preparative IEF potentially applicable to large scale purification of peptides. The core of the system is an apparatus based on a new recycling principle¹. The solution to be fractionated is continuously recycled between a multichannel heat-exchange reservoir and a multichannel focusing apparatus. Fluid flow through the focusing apparatus is stabilized by a parallel array of filter elements which streamline the flow of fluid and eliminate the problems of boundary distortions commonly observed in other types of continuous flow electrophoresis instruments. Joule heat is dissipated in the heat-exchange reservoir, rather than the focusing apparatus itself. This eliminates the throughput constraints inherent in other types of electrophoresis instruments and virtually unlimited quantities of sample can be processed.

The apparatus has been complemented by automated data collection sensors which periodically monitor the pH and ultraviolet (UV) absorption in all recirculating channels. These sensors are interfaced with a Hewlett-Packard desk top computer and plotter which provide real-time data outputs.

The usefulness of preparative IEF is at present limited by the need to employ Ampholine for the establishment of stable pH gradients. This results in product contamination with this ill-defined buffer. Several attempts have been made to develop stable pH gradients using well defined buffer systems²⁻⁴, but none has yet been quite successful. Our approach to this problem is guided by computer simulation of the isoelectric process using an explicit theory of IEF developed in our

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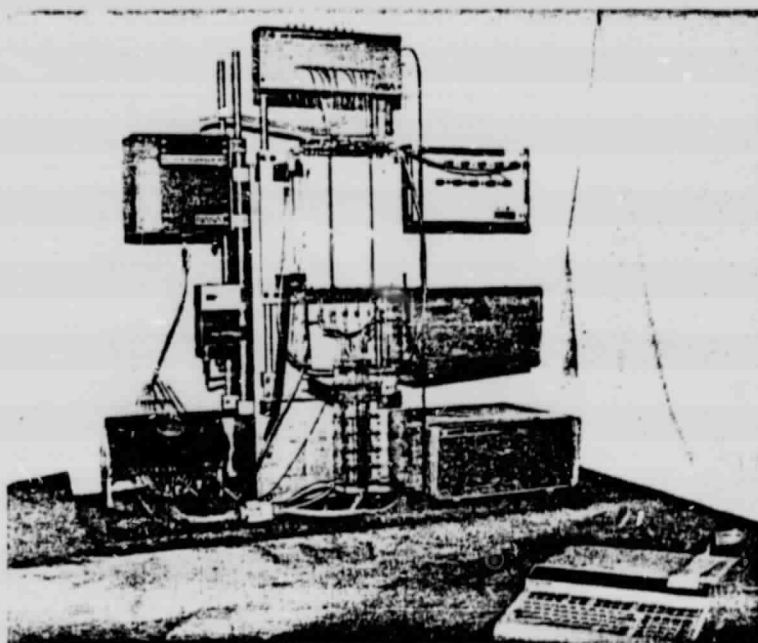


Fig. 1. Recycling isoelectric focusing apparatus (RIEF).

laboratory⁵. Careful choice of two or three ampholytes, typically amino acids or dipeptides, will generate stable gradients over a narrow pH range overlapping their isoelectric points. Several such buffer systems capable of giving useful separations are in current use.

Apparatus

A modular approach was taken in the design of the IEF system, as shown in Figure 1. The photograph illustrates a separation of two colored proteins, albumin blue stained and red hemoglobin, narrowly confined by the focusing process to two channels of the heat-exchange reservoirs. The components of the system are represented schematically in Figure 2. The system can be envisaged as being composed of two interlinked parts.

The Recycling Isoelectric Focusing Apparatus (RIEF) — comprises the essential components for the focusing process:

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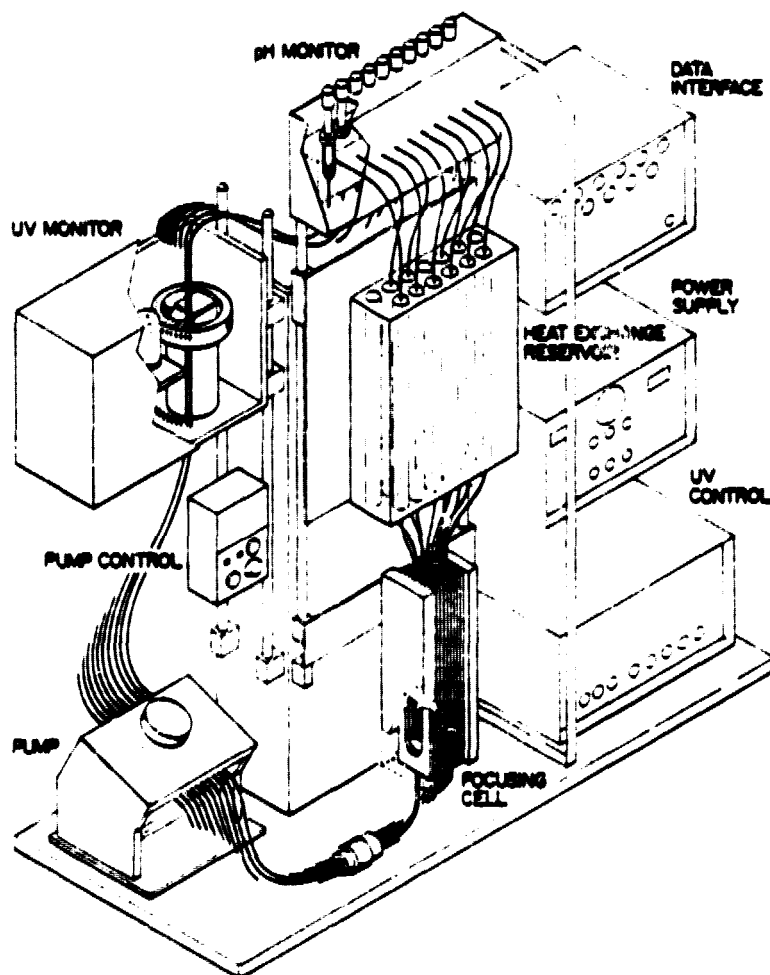


Fig. 2. Schematic presentation of the RIEF apparatus.

1. The multichannel heat-exchange reservoir is the holding container for the solution to be fractionated. It comprises 10 individual glass tubes for the sample solution, two tubes for the electrode electrolytes, and a tube for the reference solution used in the calibration of the UV monitor. Each sample tube feeds directly into a corresponding channel of the focusing cell, from where it is pumped through the UV and pH monitors and back into the same tube.

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2. The focusing cell consists of a number of narrow channels separated from each other by filter elements, the purpose of which is to streamline the flow of liquid through the cell. The cell comprises two plastic end plates with recessed platinum electrodes between which any number of spacers can be assembled. These spacers have an internal cavity of $20 \times 2 \times 0.2$ cm, coextensive with the electrodes and have means for attachment of input and output tubing. Neither the chemical nature of the filter elements nor their porosity is critical and a variety of filters are being used. In the present apparatus, we use arbitrarily an assembly of ten spacers, corresponding to the ten sample tubes in the reservoir. The spacer assembly is separated from the electrode compartments by ion-exchange membranes to avoid mixing of the sample with the anode and cathode electrolytes. The electrical transport is transversal to the downward flow of the fluid and in every pass through the cell only a small drift of solutes across the filters will suffice to gradually establish final equilibrium distribution of all components.

3. A multichannel peristaltic pump with a planetary gear drive is used for the recirculation of all fluids. The pump controller permits the regulation of the flow to about 10 ml/channel/min and the direction of flow is reversible.

The Multiplexed Electronic Data Acquisition System (MEDAS) is not an essential part of the focusing system, but was designed to facilitate data acquisition with the RIEF apparatus. It is not within the scope of this article to describe the design and construction of this part of the apparatus¹, but it will suffice to mention that it is capable of measuring periodically the UV absorption and pH in all flowing channels of the RIEF apparatus. MEDAS is under the control of a Hewlett-Packard Model 9825A desk top computer, which receives raw data from the MEDAS interface at preset time intervals, converts these raw data into optical density and pH units, performs a variety of statistical analyses, provides printouts of data in real time, and stores them on a magnetic tape for future manipulations. In addition, the computer is interfaced with a Hewlett-Packard Model 9872A x-y plotter, which is programmed to graphically display the pH, UV, current and statistical evaluation of pH and UV data in three separate plots, as is shown in Figure 3.

MEDAS can also be used for feed-back control of the separation process, as the pH gradient in the system can be manipulated by the addition or withdrawal of buffering components in the recycling appar-

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atus. Withdrawal of Ampholine from either or both of the two extreme subcompartments of the RIEF apparatus will result in a progressive flattening of the pH gradient. The computer can be programmed to establish or maintain a desired pH gradient, by relay mediated control of infusion pumps. Alternatively, if the mixture contains a major component sensed by the UV monitor, it can be made to focus into a desired channel, by similar buffer manipulations.

Operation

The computer is programmed to instruct the operator in the proper sequence of operations and requests pertinent data for entry into its memory. The monitors are calibrated first with a buffer of pH 7 with no UV adsorption, followed by a buffer of pH 4 with an OD of approximately 1. The scaling factors are automatically computed and stored in the computer memory for conversion of raw monitor data into OD and pH values. Each recorded measurement is actually the average of 25 measurements taken at close time intervals (2 msec) and the complete cycle of 10 pH, 10 UV, and 1 current measurement (actually 525 measurements) requires less than 2 sec.

Following calibration, the Ampholine containing solution (or mixture of amino acids as discussed later on) is introduced into the heat-exchange reservoir and circulation through the cell established. Air purging is accomplished by brief reversals of flow direction at rapid flow rates. It is important to emphasize that all electrophoretic transport occurs only within the focusing cell itself, across its filter elements. The pH gradient is also established within the focusing cell, by focusing of the buffer components. As the final equilibrium distribution of all components is independent of the starting distribution, the sample can be added to any or all of the channels in the heat-exchange reservoir. Nevertheless, it is advantageous to first prefocus the buffer alone, and then add a concentrated sample solution to one of the center channels. This avoids the exposure of the sample to extreme pH values, as the pH gradient is formed faster than the sample equilibrates, and minimizes the exposure of the sample.

The progress of focusing can be followed by the computer printouts and graphic displays. Usually, a set of 24 measurements at preset time intervals is taken during any focusing experiment. Mostly, 2 h total time are sufficient, and the computer will take the measurements at 5 min intervals. At the end of the experiment, all channels are collected simul-

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taneously by rechanneling of all flows into a test tube array held in an appropriate device.

Because of the modular nature of the design of the apparatus, there is great flexibility in the number of fractions collected or volume capacity. The apparatus shown has only 10 channels, but this number can be increased or decreased at will. The volume capacity of the instrument depends mainly on the volume of the heat-exchange reservoir. The reservoir shown in Figure 1 has a total capacity of 400 ml and another reservoir of 1,000 ml is also frequently used. The focusing process has been carried out also at the 10 liter level, using larger spacers with an effective area of 100 cm².

Implicit in the focusing process is the desalting of the sample, all salts ending up in the two electrode electrolytes. Precipitation of some proteins insoluble at their isoelectric point may be avoided by the addition of 8 M urea or various non-ionic detergents.

Results

The RIEF system is presently being studied for the separation of a variety of proteins and peptides, including glucagon and various synthetic derivatives, myosin peptides, synthetic ACTH, thymosin, cobra venom, hementerin, β -MSH, acid phosphatase, and others. Time will permit the description of only a few results.

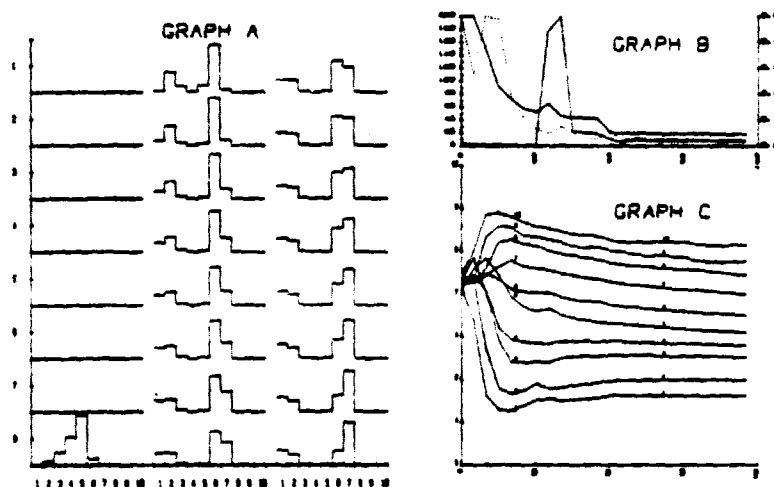


Fig. 3. MEDAS plots documenting the separation of hemoglobin and albumin (see text).

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Figure 3 exemplifies a real-time MEDAS plot obtained with two colored proteins, hemoglobin and albumin stained with bromphenol blue. Fractionation of 1 g total protein was carried out at 200 volts in 1% Ampholine, pH 3.5-10. Graph A shows 24 sequential profiles of the UV absorption across the 10 channels, taken at 5 min intervals. The first 7 profiles were flat, this marking the prefocusing of Ampholine. The protein mixture was added in channel 5, as seen from plot 8, column I. The progress of focusing can be followed by scanning column II & III, albumin finally concentrating in channels 1 & 2, hemoglobin in channels 6 & 7.

The solid line in graph B records the decrease of the focusing current with time to a final value of 18 mamp. The dotted line illustrates the variance between subsequent sets of pH measurements, and the dashed line represents the same data for UV measurements. All three lines settle down to their minimum at about 70 min focusing, inclusive of prefocusing, which indicates the equilibration of the system. Thereafter, there is only a small cathodic drift, found in all isoelectric focusing.

Graph C plots the pH values as a function of time and shows that the pH gradient is developed within the 35 min of prefocusing, followed by a slow drift towards lower pH values.

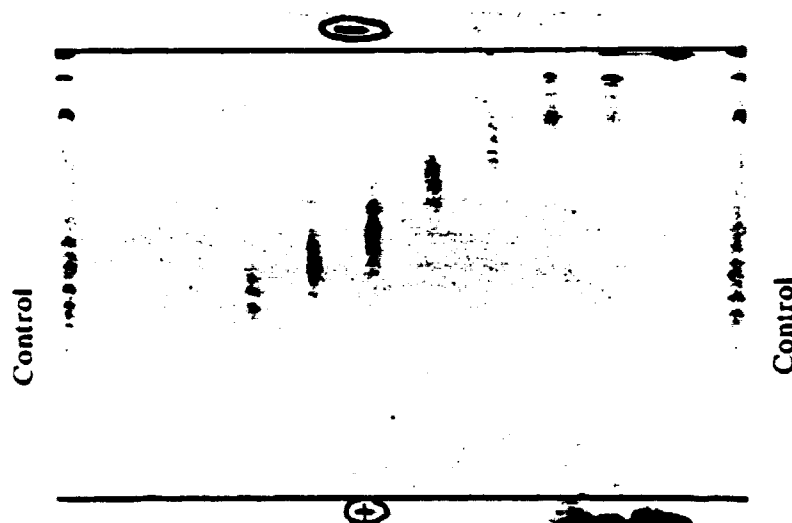


Fig. 4. RIEF fractionation of myosin peptides. Myosin CNBr peptides, RIEF 1% Ampholine, pH 3.5-7, PAG 2% Ampholine pH 3.5-7, 2/23/79

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To assess the resolution achievable, more complex systems are necessary. Peptides obtained by cyanogen bromide cleavage of myosin (250 mg) were fractionated in 400 ml of 8M urea, with 2 ml each of Ampholine, pH 3.5-5 and pH 5-7. Figure 4 shows the analytical gel patterns of the 10 RIEF fractions and of the original mixture. Excellent resolution has been obtained with only minor overlap of components between subsequent fractions.

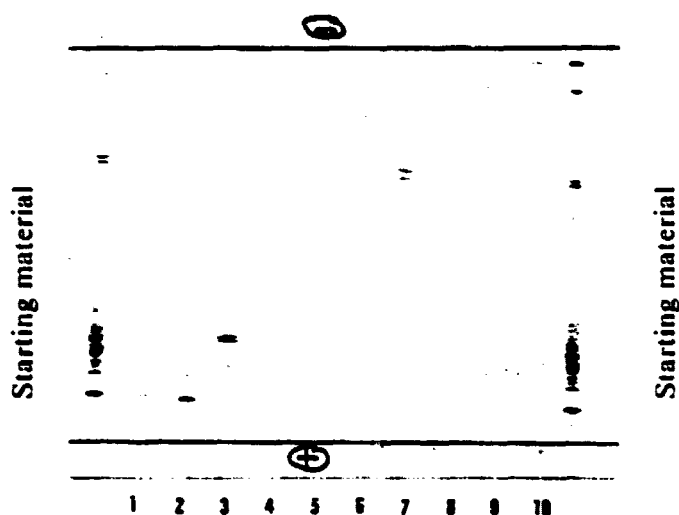


Fig. 5. RIEF fractionation of cobra venom. Cobra venom, *Agkistradon Halys Blomhofii* pH 4-6.5. PAG IEF pH 4-6.5, 4 15 79

Figure 5 presents comparable data for the fractionation of 250 mg of cobra venom in 250 ml distilled water containing 4 ml Pharmalyte, pH 4-6.5. The pH values of the ten fractions were 4.09, 4.31, 4.54, 4.78, 5.04, 5.37, 5.69, 6.01, 6.56, and 7.42. Phospholipase activity was associated with the first two fractions, and the specific activity increased by a factor of 6.2.

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Development of New Buffer Systems

IEF requires the generation of stable pH gradients, usually achieved by means of synthetic mixtures of polyaminopolycarboxylic acids, commercially available under a variety of tradenames, such as Ampholine, Pharmalyte, etc. While these are well suited for analytical purposes, they cause contamination of purified products in preparative applications. Several authors²⁻⁴ have attempted to develop stable pH gradients using simpler buffer systems of clearly defined composition.

In trying to achieve the same objective, we thought it necessary to first develop an explicit theory of isoelectric focusing of simple ampholytes describing their steady-state distribution in an electric field⁵. This mathematical model of IEF rests on the following basic concepts:

(A) the concentration of component subspecies is defined by equations of chemical equilibria; (B) in the steady state, a balance exists between the mass transports resulting from electromigration and from diffusion; and (C) the condition of electroneutrality prevails in the physical scale of the system under consideration. From the classic relationship describing these concepts and the values entered or dissociation constants of the individual components, their electrophoretic mobility, initial concentration, and current density, the model computes the pH, conductance, and concentration of each component along the axis of an assumed IEF column.

The model is too complex to be described in the present paper, but it has permitted the compilation of a library of simulated IEF runs with a variety of ampholyte mixtures. This is presently used as the basis for a rational selection of components for the establishment of stable pH gradients. These are being evaluated in three experimental systems: polyacrylamide gels, density stabilized columns and the automated RIEF apparatus. Separation of hemoglobin and albumin in the RIEF apparatus, comparable to that shown in Figure 3, is obtainable using 3 component systems with glutamine and arginine or lysine as acidic and basic ampholytes and glycyl-glycine or glycyl-glycyl-glycine as the intermediate buffering ampholyte, all in the 2.5 to 15 mM concentration range. The pH gradient is not linear, but is adequate for this separation. The proteins have to be added at the beginning of the fractionation and not after prefocusing, as otherwise the so-called conductivity gap prevents equilibration.

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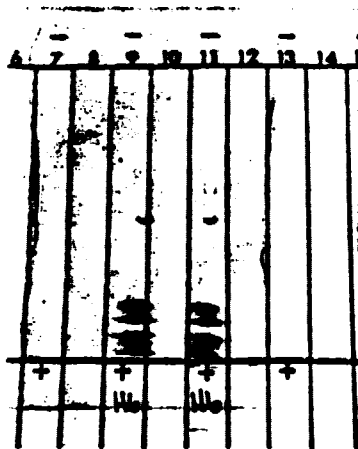


Fig. 6. Polyacrylamide gel focusing of hemoglobin in Ampholine-free buffer (see text).

Narrow pH gradients are also obtainable, and in Figure 6 is shown the focusing of hemoglobin in a polyacrylamide gel containing only 16 mM histidine (pI 7.54) and 16 mM β -alanyl-histidine (pI 8.17). The resolution of the hemoglobin bands is comparable to that obtainable with narrow pH range Ampholine. Other comparable ampholyte systems are presently under investigation.

Discussion

The RIEF system described in this paper represents a significant innovation in preparative IEF. While multicompartmented cells are among the oldest electrophoretic devices⁶, this is the first IEF apparatus in which recycling is employed. In this respect, it bears closest analogy to forced-flow electrophoresis⁷. Due to the modular nature of the apparatus, virtually unlimited quantities of proteins or peptides can be processed. Much remains to be learned about optimization of the separations, expansion of the pH scale by reprocessing of individual or pooled fractions in a second run across all ten channels, feedback control, etc. These studies are currently under way.

Acknowledgments

This work was supported in part by the NASA Grant NSG-7333 and the NASA Contract NAS8-32950. The authors wish to express their appreciation to the numerous collaborators who have contributed most of our sample materials.

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